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Structure Activity Relationship of Xanthones for Inhibition of Cyclin Dependent Kinase 4 from Mangosteen (Garcinia Mangostana L.)

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Abstract

The mangosteen fruit is a popular Southeast Asian fruit consumed for centuries. There have been a variety of xanthones isolated from the fruit, bark, roots and leaves with each having unique chemical and physical properties. Previously, the most abundant xanthone α-mangostin has been shown to inhibit CDK4. Herein we describe the role of selected xanthones from the mangosteen inhibiting CDK4. The evidence we provide here is that key functional groups are required to inhibit the CDK4 protein to prevent the phosphorylation of downstream targets critical to inhibiting uncontrolled cell cycle progression. To define the properties of xanthones for inhibiting CDK4 we utilized a cell free biochemical assay to identify inhibitors of CDK4. The following xanthones were used for the analysis: α-mangostin, β-mangostin, γ-mangostin, gartanin, 8-desoxygartanin, garcinone C and garcinone D, 9-hydroxycalabaxanthone, and 3-isomangostin These results further substantiate the unique pharmacological properties of individual xanthones and how a mixture of xanthones may be responsible for a multi-targeted effect in cell based pharmacology systems.

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Introduction

The purple mangosteen (*Garcinia mangostana L.*) is a fruit-producing tree that is native to Southeast Asia and has been used for hundreds of years for medicinal purposes [1]. All parts of the tree are used medicinally, as the roots, tree bark, and fruit are all consumed and can be commonly prepared as powders, ointments, and teas for treatment of dysentery, eczema, psoriasis, diarrhea, urinary disorders, and for healing wounds and ulcers [1, 2]. A class of biologically active compounds called xanthones can be found in all different parts of *G. mangostana*, including the outer pericarp rind, whole fruit, trunk, branches, and leaves [1]. Xanthones contain a C6-C1-C6 ring structure, with various isoprene and hydroxyl units branching off the foundational tricyclic structure. These units are thought to be important contributors to xanthones' biological activities [3]. There have been more than 70 different xanthones isolated from *G. mangostana* [3-5], with α-mangostin being the most abundant xanthone found and α-mangostin, β-mangostin, γ-mangostin, gartanin, and 8-desoxygartanin being the most studied xanthones[1]. These xanthones have been shown to have cytotoxic, anti-tumor and anti-cancer activities and here we will present data showing that xanthones inhibit cyclin dependent kinases (CDK's)[4-7].

Given the potential that xanthones have shown as promising new anti-cancer agents, we have further evaluated their role in prostate, colon, and breast cancers. In many cancers, including the three aforementioned, CDK's have become increasingly important targets, as there is often abnormal activation and upregulation of CDK's and their regulation of the cell cycle in cancer[8, 9]. CDK 4 is especially important as it controls the G1 checkpoint, where cells commit to entering the DNA synthesis phase of the cell cycle[10]. Recently, CDK 4/6 inhibitors have been tested in preclinical and clinical settings and have seen success as therapeutics in prostate and breast cancer[10, 11]. Previously, our lab has shown that α-mangostin not only inhibits the activity of CDK 4 in a cell free assay, but also decreases xenograft tumor growth in mice[7]. Here we furthered our studies of CDK 4, using eight different xanthones *in vitro*, and show them to be not only cytotoxic, but also to inhibit CDK 4 activity.

Materials and Methods

Cell Culture

HCC 1937 and MDA-MB-231were cultured in RPMI1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were maintained under standard cell culture conditions as described previously[12, 13].

Cell Viability

Cell viability of xanthones (1-100 μM) isolated from the mangosteen fruit included: α-mangostin, β-mangostin, γ-mangostin, gartanin, 8-desoxygartanin, garcinone C and garcinone D, and 3-isomangostin. Cell viability following treatment with selected xanthones was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described[7, 12]. For this assay, 10^4 and $2x10^4$ cells of HCC1937 and MDA-MB-231 cell lines were plated in 96 well plate and incubated for 24h. Following incubation, media was replaced with media containing the test compounds in serial dilution ranging from 100 μM to 1 μM in duplicate. After incubating the cells with test compounds for 48 h, test media was replaced with media containing MTT reagent (Sigma-Aldrich) at a final concentration of 0.5 mg/mL. The cells were incubated for a period of 2 h and the media was discarded and the formazan crystals were solubilized in DMSO on a plate shaker protected from light at all times. The reading was taken at a wavelength of 570 nm and the viability was calculated with respect to control (0.01% DMSO). IC_{50} was calculated with Graphpad Prism 5.0. using Non-linear regression analysis.

CDK4 Inhibition Assay

A cell free assay was utilized as previously described.[7] Xanthones were diluted into the assay mixture with concentrations ranging from 10 to 40 μM of xanthones to determine inhibition of CyclinD1/CDK4 (Kinexus, Vancouver, British Columbia, Canada). The IC_{50} for each xanthone was determined against CyclinD1/CDK4. The addition of [33P]-ATP to the reaction mixture initiated the reaction. Following room temperature incubation the assay was terminated by spotting 10 μl of the reaction mixture onto multiscreen phosphocellulose P81 plate and washed three times for ~15 min each in a 1% phosphoric acid solution. The

radioactivity on the P81 plate was counted in the presence of scintillation fluid in a Trilux scintillation counter. Protein kinase-specific activity of [33P]-ATP incorporated per minute per sample was determined. A total counts per minute for each reaction sample was determined for blanks (without substrate), control (without α-mangostin) and samples (with α-mangostin). The corrected activity for control samples (i.e. without α-mangostin) represented 100% kinase activity and was used to determine the percent of kinase activity.

Results

Selected Xanthones Inhibit HCC1937 and HCT116 Cancer Cells

A decrease in cell viability was observed following treatment of individual xanthones to HCC-1937 cells and HCT116 cells (Table 1). Following a 48 hour treatment with xanthones the IC_{50} (μM) for cell viability was determined for α-mangostin (13.0), β-mangostin (18.6), γ-mangostin (14.4), gartanin (31.2), 8-desoxygartanin (24.4), garcinone C (38.6), garcinone D (32.2), and 3-isomangostin (59.9). The colon cell line HCT 116 was treated with increasing concentrations of xanthones (0 – 100 μM) with the following EC_{50} calculated for each α-mangostin (23.4), β-mangostin (19.1), γ-mangostin (12.1), gartanin (6.84), 8-desoxygartanin (18.2), garcinone C (27.4), garcinone D (15.8), and 3-isomangostin (47.4).

Selected Xanthones Inhibit CDK4/Cyclin D1 in a Cell Free Biochemical Assay

To determine if xanthones inhibited CDK4/ Cyclin D1 in a cell free assay CDK4/Cyclin D1 was exposed to individual phytochemicals to determine if phosphorylation of the substrate was inhibited (Table 2 and Figure 1).

Inhibitory activity as determined by an IC_{50} was determined for the following xanthones: α-mangostin (8.5 μM), γ-mangostin (6.2 μM), garcinone D (12.8 μM), and 3-isomangostin (15.6 μM). Several xanthones did not display any significant inhibition of CDK4/Cyclin D1 included: β-mangostin, garcinone C, gartanin, and 8-desoxygartanin.

Discussion

Deregulation of the CDK4/cyclin D1-retinoblastoma (Rb) pathway is an important contributor to increased mitogenic potential of cancer cells[14]. Further, there is evidence suggesting that deregulation of this pathway contributes to endocrine therapy resistance in hormone dependent cancers including breast cancer[15]. Cyclin dependent kinase activity is regulated by several variables that include T-loop phosphorylation, abundance of cyclins, and association with CDK inhibitors (Cip/Kip) and/or INK family proteins[16]. The active complex of CDK4 is CDK4/ Cyclin D1 that targets the retinoblastoma protein for phosphorylation. This post-translational modification releases E2F transcription factors that activate G1/S phase gene expression.

α-Mangostin represents the most abundant xanthone in the mangosteen and by default makes it the most studied xanthone. The mangosteen has more than 80 xanthones isolated from the whole plant suggesting a significant degree of chemical diversity may be responsible for its multi-targeted effect in cancer models.[17, 18] Taken together this approach emphasizes the need to evaluate a variety of xanthones to more adequately describe the pharmacological properties of a complex extract of xanthones. Previously, we have described the inhibitory properties of α-mangostin against CDK4 using a combination of cell free biochemical, cell based models, *in vivo* animal model along with molecular modeling[7].

At present our working hypothesis is that α-mangostin and more specifically the isoprenyl group at 2 position is able to bind deep within the ATP binding pocket (Figure 2)[7]. It is possible that the other end of α-mangostin (i.e. carbons 5-8) is able to bind into the pocket, however, we consider this unlikely in that the pocket does not fill as effectively. Interestingly, we have previously shown that gartanin is a potent AR disruptor, however, it does not inhibit CDK4[7]. Our data shown in Figure 1 interestingly, shows that when an isoprenyl group is at the 4 position (e.g. gartanin and 8-desoxygartanin) and is absent at the 8 position there is no inhibition of CDK4. Based on these results it could be that when an isoprenyl group is present at the 4 position (e.g. gartanin and 8-desoxygartanin), this prevents the xanthone from binding the ATP binding pocket of CDK4. Hydroxylation of key groups also appears to be important as shown in Figure 1A when

Table 1. IC_{50} (μM) of selected xanthones from *Garcinia mangostana* (mangosteen) against HCC1937 and HCT116

Xanthone	HCC 1937	HCT 116
α-mangostin	13.0	23.4
β-mangostin	18.6	19.1
γ-mangostin	14.4	12.1
garcinone c	38.6	27.4
garcinone d	32.2	15.8
3-isomangostin	59.9	47.4
gartanin	31.2	6.8
8-desoxygartanin	24.4	18.2

Cells were exposed to increasing concentrations of xanthone (0-100 uM) for 48 hours in triplicate. A mean and standard deviation was determined for each to establish the EC50. This was followed by determining the cell viability using an MTT assay.

Table 2. Inhibitory activity of xanthones from *Garcinia mangostana* (mangosteen) against CDK4/Cyclin D1

	% Activity					
Xanthone	Control	10 μM	20 μM	30 μM	40 μM	EC50
α-mangostin	100 ± 1.38	41.29 ± 4.01	15.81 ± 1.89	13.05 ± 0.44	8.77 ± 0.42	8.5
β-mangostin	100 ± 1.38	91.7 ± 3.40	78.60 ± 1.94	64.83 ± 5.67	54.97 ± 2.88	–
γ-mangostin	100 ± 1.38	20.01 ± 2.38	6.10 ± 1.09	0.59 ± 0.72	0.51 ± 0.55	6.2
garcinone C	100 ± 1.38	100.92 ± 4.91	86.07 ± 10.50	75.11 ± 0.85	65.65 ± 7.94	–
garcinone D	100 ± 1.38	65.38 ± 5.33	12.89 ± 1.52	9.72 ± 0.39	9.02 ± 0.58	12.8
9-hydroxycalabaxanthone	100 ± 1.38	83.91 ± 5.18	63.14 ± 4.70	49.65 ± 3.88	83.53 ± 3.08	30.0
3-isomangostin	100 ± 1.38	83.53 ± 3.72	24.15 ± 2.48	10.77 ± 1.25	7.4 ± 1.38	15.6
gartanin	100 ± 1.38	99.63 ± 0.14	97.85 ± 11.61	92.9 ± 3.75	80.21 ± 10.25	–
8-desoxygartanin	100 ± 1.38	97.25 ± 4.66	93.46 ± 6.45	82.87 ± 1.13	81.42 ± 6.38	–

Values represent the mean of three values followed by the standard deviation.

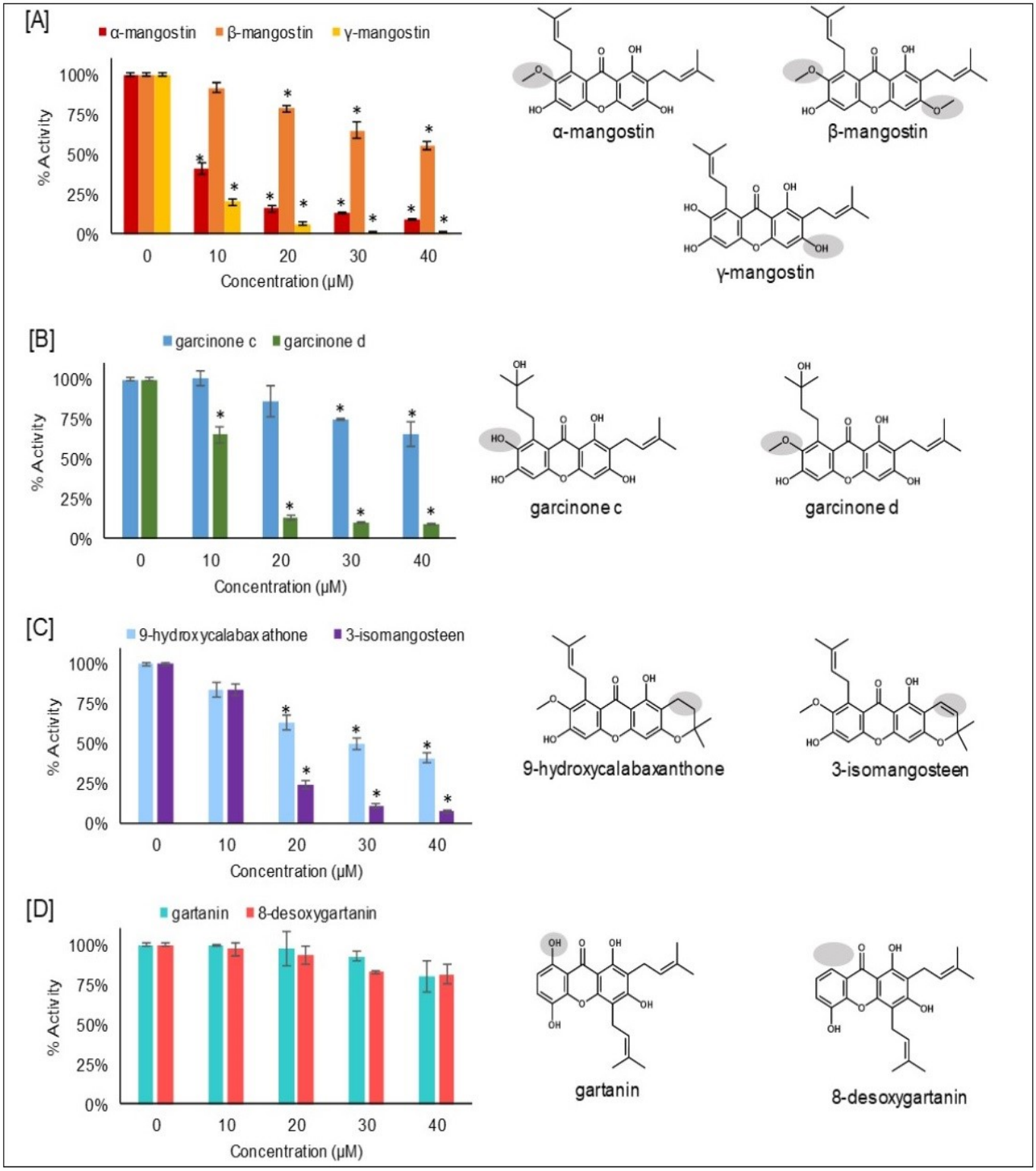

Figure 1. Cell free biochemical kinase assay established the IC50 of x anthones against CyclinD1/CDK4. Data points are represented by the average of three values with standard deviation. Statistical analysis was performed using GraphPad software by one way analysis of variance and Statistical significance was performed by the Tukey test with *P < 0.01. All values are compared to the control sample.

comparing the CDK4 inhibition profiles of α-mangostin, β-mangostin, and γ-mangostin. Hydroxylation at the 3 and 7 positions provided the most potent of the three (i.e. γ-mangostin compared to α-mangostin and β-mangostin). Unfortunately, the experiments performed in a cell free biochemical assay do not consider phase I metabolism which may occur. In an *in vivo* model or real world setting it could be possible that methoxy groups such as those present in α-mangostin (i.e. 7 carbon), β-mangostin (i.e. 3 and 7 carbons) may undergo o-demethylation to generate γ-mangostin. When comparing γ-mangostin to garcinone D it is evident that the when the isoprenyl group is hydroxylated it can counter the methoxy group at the 7 position ultimately restoring CDK4 inhibition. We have described previously that α-mangostin undergoes Phase II metabolism to form mono-glucuronide and di-glucuronides after oral administration to mice.[19, 20] More work is needed to understand the potential of o-demethylation of xanthones from the mangosteen following exposure to phase I enzymes (P450). This is especially significant as most individuals who consumer mangosteen will be exposed to a variety of xanthones.

Conclusion

Taken together we provide an explanation as to how α-mangostin inhibits CDK4 using an additional 8 xanthones. Key functional groups appear to be the isoprenyl groups at the 2 and 8 positions with hydroxyl groups extending from 3 and 7 positions. Another important consideration is the lack of an isoprenyl group at the 4 position for inhibition of CDK4. Interestingly, we have shown that gartanin does not inhibit CDK4 while inhibiting AR functionality while α-mangostin inhibits CDK4. Future studies are clearly needed to characterize the key functional groups needed for specific pharmacological actions of xanthones isolated from the mangosteen fruit.

Conflicts of Interest

The authors do not declare any conflicts of interest.

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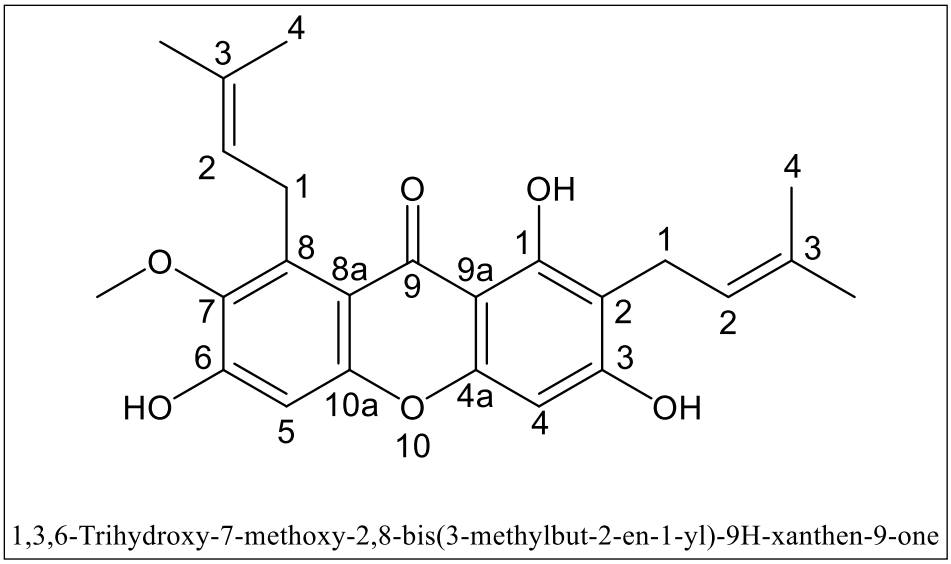

1,3,6-Trihydroxy-7-methoxy-2,8-bis(3-methylbut-2-en-1-yl)-9H-xanthen-9-one

Figure 2. α - Mangostin

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